Analytical Methods

Deutsche Ausgabe: DOI: 10.1002/ange.201609178
Internationale Ausgabe: DOI: 10.1002/anie.201609178

Excited Fluorophores Enhance the Opening of Vesicles at Electrode Surfaces in Vesicle Electrochemical Cytometry

*Neda Najafinobar, Jelena Lovrić, Soodabeh Majdi, Johan Dunevall, Ann-Sofie Cans, and Andrew Ewing**

Abstract: *Electrochemical cytometry is a method developed recently to determine the content of an individual cell vesicle. The mechanism of vesicle rupture at the electrode surface involves the formation of a pore at the interface between a vesicle and the electrode through electroporation, which leads to the release and oxidation of the vesicle's chemical cargo. We have manipulated the membrane properties using excited fluorophores conjugated to lipids, which appears to make the membrane more susceptible to electroporation. We propose that by having excited fluorophores in close contact with the membrane, membrane lipids (and perhaps proteins) are oxidized upon production of reactive oxygen species, which then leads to changes in membrane properties and the formation of water defects. This is supported by experiments in which the fluorophores were placed on the lipid tail instead of the headgroup, which leads to a more rapid onset of vesicle opening. Additionally, application of DMSO to the vesicles, which increases the membrane area per lipid, and decreasing the membrane thickness result in the same enhancement in vesicle opening, which confirms the mechanism of vesicle opening with excited fluorophores in the membrane. Light-induced manipulation of membrane vesicle pore opening might be an attractive means of controlling cell activity and exocytosis. Additionally, our data confirm that in experiments in which cells or vesicle membranes are labeled for fluorescence monitoring, the properties of the excited membrane change substantially.*

Electrochemical techniques can be used to detect and quantify the neurotransmitter content in soft nanoparticles, such as vesicles or liposomes, by electrochemically oxidizing transmitters upon particle rupture.[1] Previous work in quantifying the neurotransmitter content of single secretory vesicles has been performed using a technique known as electrochemical cytometry.[1f] This technique involves the separation of vesicles or liposomes using capillary electrophoresis followed by amperometric detection of their content as the vesicles exit the capillary and are lysed at the surface of a carbon fiber microelectrode. We have recently developed a technique with the same aim, vesicle electrochemical cytometry, that involves the lysis of vesicles on an electrode surface without the need for a preceding electrophoretic separation step.[2] The electroactive content of an individual vesicle is oxidized at the electrode surface and results in an amperometric spike. The area under the spike provides the number of molecules detected, while the shape characteristics of the spike reveal kinetic information of the opening of the vesicle at the electrode. The number of amperometric spikes also relates to the number of vesicles opening at the electrode surface. Application of a potential at the electrode surface appears to cause electroporation of the membrane and leads to the formation of a pore between the electrode and vesicle, out of which the neurotransmitters can diffuse.[3] Not all adsorbed vesicles form a pore and, interestingly, in most cases vesicles stay intact.[4] However, if we replace the vesicles with liposomes, as a model for secretory vesicles, the number of liposomes that rupture at the electrode surface increases significantly compared with that of vesicles.[3] This gives rise to the important question of which factors affect the adsorption of vesicles at the electrode surface and the initial formation of the pore.

The ability to manipulate vesicle opening on electrodes or in cells is extremely important, as this is part of the fundamental process of communication, and might be a means to control cell activity and communication with light and without the involvement of channel proteins. Furthermore, the concept that oxidative stress affects the opening of membrane pores is of fundamental importance in our understanding of membrane dynamics.

Many studies on the adsorption and rupture pathways of liposomes have shown that both the adsorption and the rupture mechanism are dependent on many factors including the lipid composition of the liposomes and the nature of the adsorption substrate.[5] In our system, we use large dense core vesicles (LDCV) isolated from chromaffin cells, which makes the interpretation of the results more challenging owing to the complex nature of the LDCVs.

Herein, we show that manipulating the membrane properties of vesicles with an excited lipid-conjugated fluorophore can increase the number of amperometric events, which indicates a significant increase in vesicle opening at the electrode surface as measured by vesicle electrochemical cytometry. The enhancement of electrochemical cytometry using fluorophores is suggested to result from oxidation of the

[*] Dr. S. Majdi, Prof. A. G. Ewing
Department of Chemistry and Molecular Biology
University of Gothenburg
Kemivägen 10, 41296 Gothenburg (Sweden)
E-mail: andrew.ewing@chem.gu.se
N. Najafinobar, J. Lovrić, J. Dunevall, Prof. A.-S. Cans, Prof. A. G. Ewing
Department of Chemistry and Chemical Engineering
Chalmers University of Technology
Kemivägen 10, 41296 Gothenburg (Sweden)
E-mail: andrewe@chalmers.se
Supporting information and the ORCID identification number(s) for the author(s) of this article can be found under: http://dx.doi.org/10.1002/anie.201609178.

membrane, which allows easier adsorption and rupture and is dependent on the location of the fluorophore within the membrane structure, that is, either at the head or tail of the vesicle lipids.

When a 33-μm electrode is placed in a suspension of isolated chromaffin vesicles and a potential of 700 mV versus Ag/AgCl reference electrode is applied at the electrode surface, the vesicles adsorb onto the electrode surface and undergo fusion over time resulting in amperometric spikes (Figure 1 a). When vesicles are labeled with a fluorophore, such as rhodamine phosphatidylethanolamine (Rh-PE), incorporated in their membrane and the fluorophore is excited, the amperometric trace shows a significant increase in the number of spikes compared to those without the fluorophore (Figure 1 b). A comparison between the number of vesicle fusion events with and without fluorophore-labeling reveals a significant increase in the number of events when the fluorophores in the labeled vesicles are excited (Figure 1 c).

The increase in the number of events could be related to a larger number of vesicles adsorbed at the electrode surface, promotion of initial pore formation, or both. We compared the kinetic parameters between control and labeled vesicles and found no significant difference in the kinetics for chemical release from individual vesicle events between the two systems (Supporting Information, Figure S1). Because of the considerably slow diffusion rate of neurotransmitters inside the vesicle matrix owing to the presence of the dense core, changing the membrane properties of the vesicle does not influence the kinetic parameters significantly. This means that the most important feature to determine the kinetic parameters for vesicle rupture is the diffusion of neurotransmitters through the pore and not the membrane properties.[3]

To determine if the enhancement in vesicle opening is dependent on the amount of dye incorporated into the membrane we incubated vesicles with different dye concentrations. The results clearly show a dependence of event frequency on dye concentration. Thus, higher dye concentrations result in more vesicles adsorbing and/or rupturing at the electrode surface (Figure 2). As expected, the same pattern was observed for different light intensities. The highest light intensity produced the largest number of spikes in an amperometric trace (Supporting Information, Figure S2). Moreover, in a different approach, the light was turned on and off in a systematic way, and the number of events increased and decreased correspondingly (Supporting Information, Figure S3), which clearly shows that exciting the fluorophore enhances the opening of vesicles.

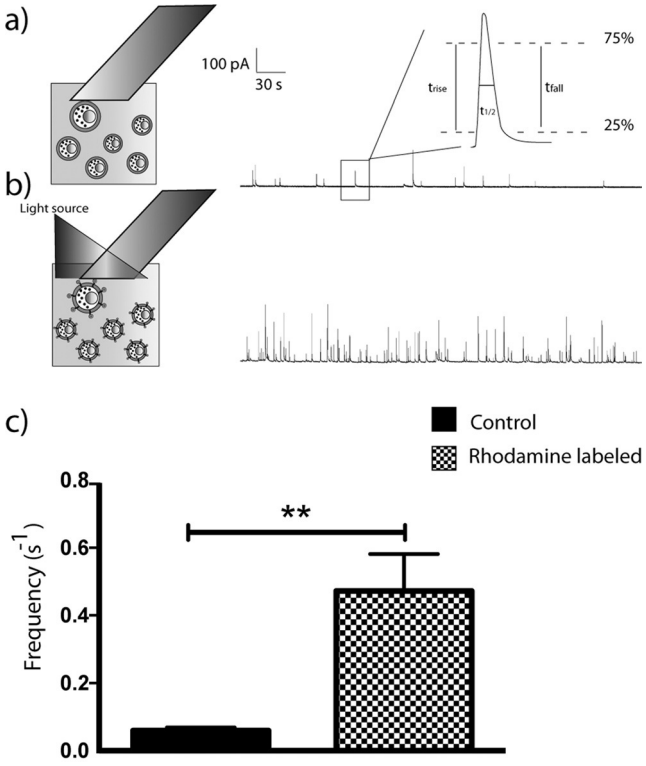

Figure 1. A schematic of the experiment with an example of an amperometric trace of vesicles opening at the electrode surface for a) control and b) rhodamine-labeled vesicles. c) Bar graph comparing frequency of events between control and rhodamine-labeled vesicles (3 μM). Error bars are the standard error of the mean; vesicle isolation was done three times; and for each isolation, three replicate experiments were performed for each condition. The total number of spikes for control and rhodamine-labeled vesicles is 358 and 2500, respectively. $^{**}p < 0.005$.

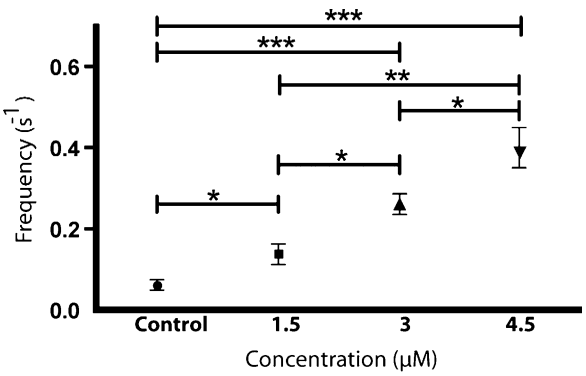

Figure 2. The frequency of events is dependent on the concentration of dye. The vesicles were labeled using 1.5, 3, and 4.5 μM rhodamine prior to the measurement; error bars are the standard error of the mean. Vesicle isolation was done three times; and for each isolation, three replicate experiments were performed for each dye concentration. The total number of spikes (from low to high concentration of dye) is 334, 626, 1271, and 2094. $^{*}p < 0.05$, $^{**}p < 0.005$, and $^{***}p < 0.001$.

An important question is whether this phenomenon is specific to rhodamine or if any excited fluorophore can produce the same effect. We tested another frequently used dye-conjugated lipid, nitro benzoxadiazol-phosphoethanolamine (NBD-PE), that has a shorter maximum excitation wavelength (460 nm) than that of rhodamine. Like Rh-PE, NBD-PE has the fluorophore attached to the lipid headgroup and therefore the fluorophore is located in the outer part of the membrane. Our data clearly show that the number of the spikes increases significantly when vesicles are labeled with NBD-PE (Figure 3) and the kinetic parameters for individual spikes are the same (Supporting Information, Figure S4).

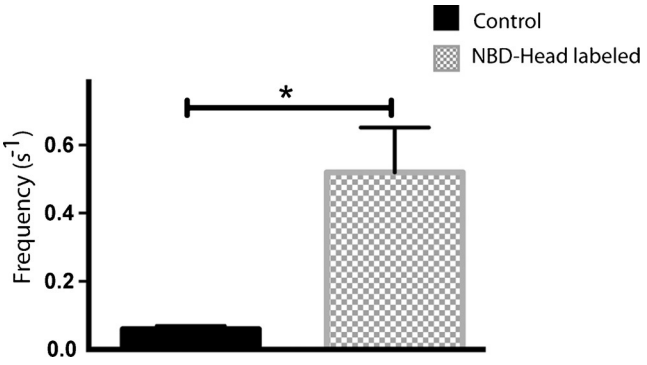

Figure 3. Bar graph showing the frequency of vesicle opening events for control and NBD-head-labeled vesicles (3 μM). The error bars are the standard error of the mean. Vesicle isolation was done three times; and for each isolation, three replicate experiments were performed for each condition. The total number of spikes for control and NBD-head-labeled vesicles is 358 and 1871, respectively. $^*p < 0.05$.

The number of spikes has been plotted versus time for comparison of control and labeled vesicles. Interestingly, the vesicles labeled with rhodamine and NBD show a rise in the frequency of events in the beginning of the recording that is followed by an exponential decay (Figure 4b,c), whereas for the control vesicles, the frequency of rupture begins at the maximal value followed by an exponential decay (Figure 4a). The initial rise in the frequency of events for labeled vesicles appears to result from the absorbed energy promoting vesicle absorption and/or pore formation upon radiation. For NBD there is a time delay before the initial rise compared with that of rhodamine, and this might be explained by the greater distance between the absorption center and the center of the bilayer, as NBD is more polar. This could reduce the effect of the fluorescent label and cause the observed time delay.[6] To test this, we used another NBD-PE dye in which the fluorophore is attached to the tail of the lipid and found the response time to be similar to the control (that is, there was no time delay to the maximal response; see Figure 4d and the Supporting Information, Figure S5). In this case, the fluorophore is located in the center of the bilayer,[7] where the reaction of excited fluorophore with the membrane might be more potent. The fact that a fluorophore placed in the middle of the bilayer is more potent for pore formation suggests that ROS formation rather than vesicle adsorption might be the more dominant factor affecting the frequency of rupture at the electrode surface.

It has been shown that the effects of excited fluorophores placed in the membrane of vesicles to promote the leakage of the encapsulated dye are dependent on the concentration of the fluorophore in the membrane.[5d,8] The fluorophore, which is attached to the lipid headgroup, is thought to be the nucleation center for pore formation or it might increase the membrane tension. In either case, this facilitates pore formation. However, different control experiments in which unlabeled vesicles were exposed to light (560 nm) and labeled vesicles were kept in the dark during measurement, result in no increase in the number of vesicle impact events (Supporting Information, Figure S6). This strongly suggests that the excited dye changes the properties of the membrane, apparently leading to better vesicle adhesion and/or easier pore formation.

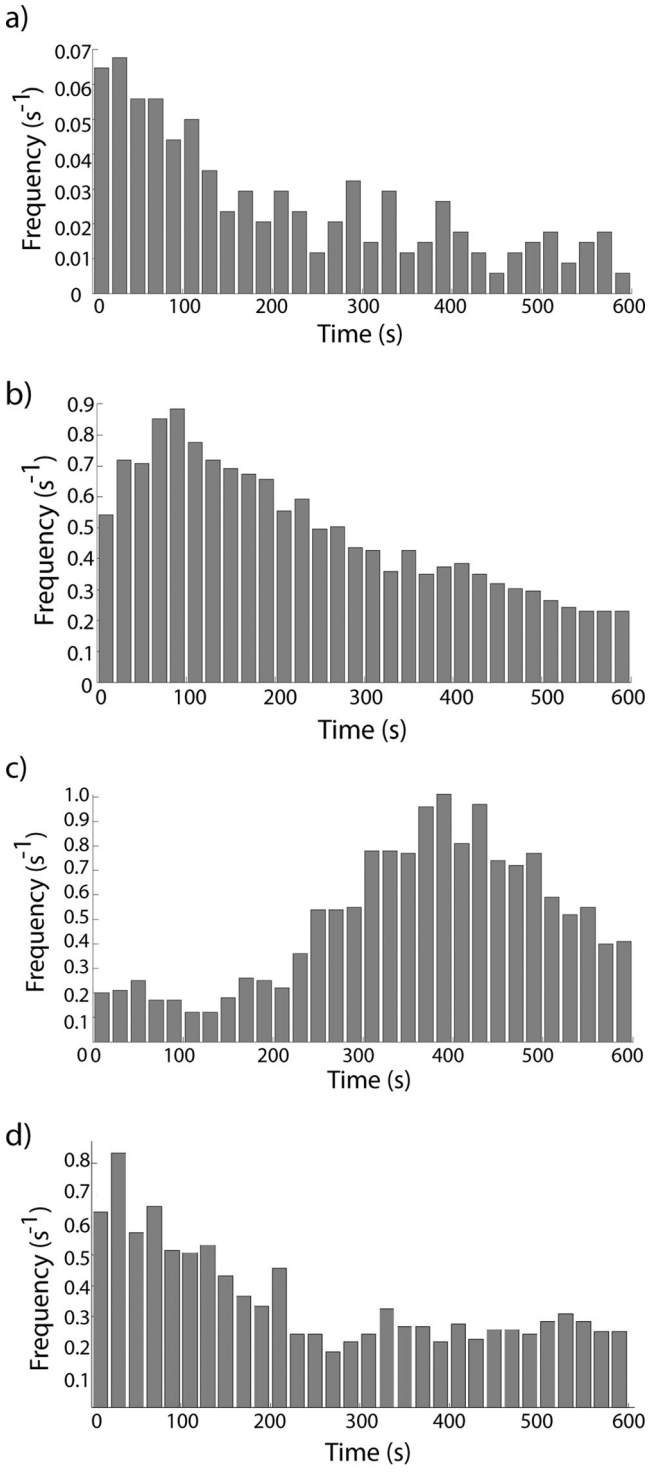

Figure 4. The bar graphs are frequency versus time for a) control, b) rhodamine-head-labeled (3 μM), c) NBD-head-labeled (3 μM), and d) NBD-tail-labeled (3 μM) vesicles.

Excited fluorophores produce reactive oxygen species (ROS) at the lipid bilayer and can cause oxidation of the membrane.[9] The production of ROS is mainly dependent on

the photochemical properties of the fluorophore and the dose of excitation light.[9] It appears that in our case the lipid attached to the dye brings the fluorophore close to the membrane, and, upon radiation, the excited fluorophore produces both singlet oxygen and superoxide, which oxidize the membrane lipids and proteins, damage the membrane, and cause a significant change in the conformation of lipids and proteins. Oxidized lipid tails become more polar and bend towards the polar lipid headgroups, leading to an increase in the average surface area per lipid and decreasing bilayer thickness. This conformational change causes the formation of water defects in the membrane bilayer and an increase in membrane permeability that is greater as the concentration of the oxidized lipids is increased. All these changes in the membrane properties can enhance the probability of pore formation. It has been shown that pore formation through electroporation occurs at local concentrations of oxidized lipids because the first steps for electroporation involve the intrusion of water into the bilayer interior and bilayers containing oxidized species are more permeable to water.[10]

Changing the permeability of the membrane also disturbs the protein localization.[11] Moreover, proteins are the main biological targets for ROS because of their abundance and high rate constants for protein oxidation, which can lead to unfolding or conformational changes of the proteins.[12] These phenomena can lead to better adsorption of vesicles onto the surface of the electrode where the lipid bilayer contacts the surface as proteins diffuse away from the contact point, leading to more effective electroporation.

The same phenomenon has been observed in other studies in which rhodamine-conjugated peptides bind to negatively charged phospholipids and, upon radiation, destabilize the membrane.[13] The excited fluorophore causes the oxidation of the membrane through production of ROS at the lipid bilayer. However, the formation of ROS alone is not enough to destroy the membrane, and the conjugated peptide plays an important role in bringing the dye close to the membrane and promoting the aggregation of oxidized lipids, which accelerate the membrane rupture.[13] In our work, lipid conjugation appears to decrease the thickness of the membrane and bring it closer to the electrode, which promotes electroporation. Thus, the proximity of the excited dye to the membrane is critically important to drive this process. This is supported by the more rapid onset of the effect in the experiments with the NBD-tail-labeled lipid in which the oxidation reaction is inside the membrane and can more effectively drive this process from the inner membrane.

To test this hypothesis, we treated chromaffin cell vesicles with dimethyl sulfoxide (DMSO) because it has the same effect on the membrane as oxidized lipids in terms of membrane thickness and formation of water defects.[14] DMSO increases the membrane area per lipid and decreases the membrane thickness, leaving more free space in the hydrophobic region of the membrane and leading to less-packed lipid tails that show disordered configurations.[14] It also causes the formation of membrane defects made by the presence of water molecules within the lipid bilayer similarly to oxidized lipids. There is also evidence that DMSO interacts with membrane proteins, breaking them into subunits.[14,15] We have treated vesicles with DMSO, and subsequent electrochemical cytometry measurements show differences from control experiments that follow the same pattern as fluorescently labeled headgroup vesicles. The number of events increases significantly (Figure 5a), while all the kinetic parameters remain the same (Supporting Information, Figure S7). The increase in the frequency of events with time observed for rhodamine- and NBD-headgroup-labeled vesicles is also observed for DMSO-treated vesicles (Figure 5b). It has been shown that electroporation efficiency in cells increases after DMSO incubation.[16]

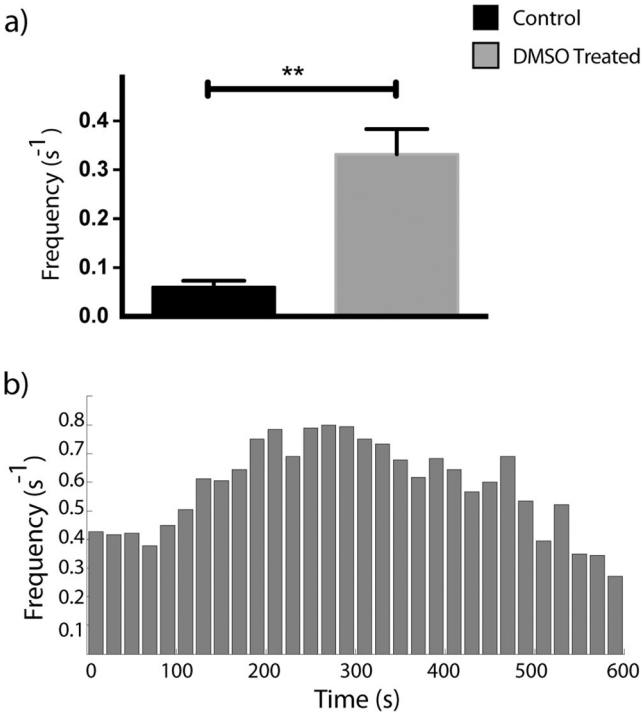

Figure 5. a) Comparison of the frequency of events between control and 0.6% DMSO-treated vesicles shows a significant increase in the frequency of spikes for the DMSO-treated vesicles. Vesicle isolation was done three times; and for each isolation, three replicates were performed for each condition. The total number of spikes for control and DMSO-treated vesicles is 326 and 1791, respectively. $^{**}p < 0.005$. b) Frequency versus time graph for opening of DMSO-treated vesicles.

To confirm that the production of ROS causes the enhancement of vesicle opening, vesicles were treated with hydrogen peroxide (H_2O_2). The data clearly shows that the number of events increases significantly in the presence of H_2O_2 (Supporting Information, Figure S8). We also loaded vesicles with the intravesicular dye acridine orange, which increased the number of events as well (Supporting Information, Figure S9a); however, a portion of the excited dye is in contact with the membrane and might well be involved in the oxidation of membrane species. Thus, this does not provide mechanistic information. The increased frequency versus time reaches a maximum at about the same time as that for the rhodamine-labeled vesicles (Supporting Information, Figure S9b), which is consistent with labeling the headgroups

but in this case the effect occurs through contact with the inside of the lipid bilayer. We note that these factors do not affect the dynamics of the individual events, even though both should affect the entire membrane. Taken together with the potential dependence of the incidence of pore formation,[3] we argue that fluorophore excitation, DMSO, and H_2O_2 promote the electroporation process to more rapidly initiate pore formation at the interface between adsorbed vesicles and the electrode, but the exact mechanism of opening can still be debated.

Figure 6 shows the proposed model of vesicle-opening enhancement with excited fluorophores. We hypothesize that this is similar for treatment with DMSO. In the model, the membrane lipids (and perhaps proteins) are oxidized by the excited fluorophore, leading to a more disrupted membrane and both better adsorption of vesicles to the electrode surface and an electroporation-formed fusion pore. It is very likely that by treating the vesicles with DMSO or by introducing excited fluorophores into the membrane we are creating a more fragile membrane, making electroporation more efficient and promoting the incidence of pore formation for opening of the vesicle in electrochemical cytometry.

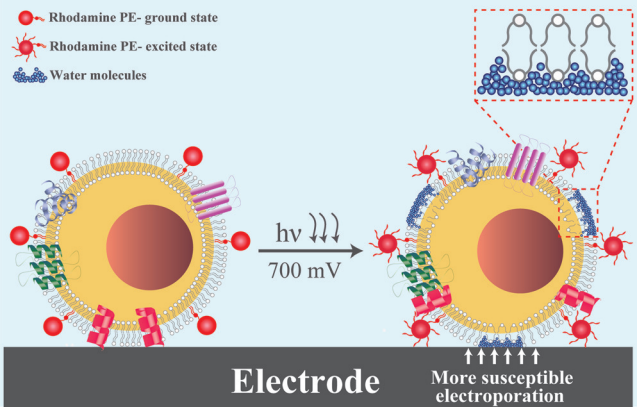

Figure 6. Model of the hypothesis that the oxidation of membrane lipids, upon production of ROS, changes the packing density of lipid tails and causes the formation of water defects followed by proteins delocalization, as suggested in a previous model.[3] The decrease in membrane thickness, formation of water defects, and protein delocalization make the vesicle membrane more susceptible to electroporation, which is a mechanistic part of vesicle electrochemical cytometry.

Acknowledgements

The European Research Council (Advanced Grant), Knut and Alice Wallenberg Foundation, the Swedish Research Council (VR), and the National Institutes of Health are acknowledged for financial support.

Keywords: electroporation · chemical cytometry · fluorophores · fusion pores · vesicles

How to cite: *Angew. Chem. Int. Ed.* **2016**, *55*, 15081–15085
Angew. Chem. **2016**, *128*, 15305–15309

[1] a) W. Cheng, R. G. Compton, *Angew. Chem. Int. Ed.* **2014**, *53*, 13928–13930; b) B. K. Kim, A. Boika, J. Kim, J. E. Dick, A. J. Bard, *J. Am. Chem. Soc.* **2014**, *136*, 4849–4852; c) E. Lebègue, C. M. Anderson, J. E. Dick, L. J. Webb, A. J. Bard, *Langmuir* **2015**, *31*, 11734–11739; d) D. M. Omiatek, A. J. Bressler, A. S. Cans, A. M. Andrews, M. L. Heien, A. G. Ewing, *Sci. Rep. UK* **2013**, *3*, 1–6; e) D. M. Omiatek, Y. Dong, M. L. Heien, A. G. Ewing, *ACS Chem. Neurosci.* **2010**, *1*, 234–245; f) D. M. Omiatek, M. F. Santillo, M. L. Heien, A. G. Ewing, *Anal. Chem.* **2009**, *81*, 2294–2302.
[2] J. Dunevall, H. Fathali, N. Najafinobar, J. Lovric, J. Wigstrom, A. S. Cans, A. G. Ewing, *J. Am. Chem. Soc.* **2015**, *137*, 4344–4346.
[3] J. Lovric, N. Najafinobar, J. Dunevall, S. Majdi, I. Svir, A. Oleinick, C. Amatore, A. Ewing, *Faraday Discuss.* **2016**, 1–20.
[4] X. C. Li, S. Majdi, J. Dunevall, H. Fathali, A. G. Ewing, *Angew. Chem. Int. Ed.* **2015**, *54*, 11978–11982.
[5] a) K. Dimitrievski, B. Kasemo, *Langmuir* **2008**, *24*, 4077–4091; b) R. Dimova, *Adv. Colloid Interface* **2014**, *208*, 225–234; c) E. Reimhult, B. Kasemo, F. Hook, *Int. J. Mol. Sci.* **2009**, *10*, 1683–1696; d) H. Schönherr, J. M. Johnson, P. Lenz, C. W. Frank, S. G. Boxer, *Langmuir* **2004**, *20*, 11600–11606.
[6] a) F. S. Abrams, E. London, *Biochemistry* **1992**, *31*, 5312–5322; b) K. Kachel, E. Asuncion-Punzalan, E. London, *Biochim. Biophys. Acta Biomembr.* **1998**, *1374*, 63–76.
[7] a) A. Chattopadhyay, *Chem. Phys. Lipids* **1990**, *53*, 1–15; b) H. Raghuraman, S. Shrivastava, A. Chattopadhyay, *Biochim. Biophys. Acta Biomembr.* **2007**, *1768*, 1258–1267.
[8] a) J. M. Johnson, T. Ha, S. Chu, S. G. Boxer, *Biophys. J.* **2002**, *83*, 3371–3379; b) P. Lenz, J. M. Johnson, Y. H. M. Chan, S. G. Boxer, *Europhys. Lett.* **2006**, *75*, 659–665.
[9] R. A. Hoebe, C. H. Van Oven, T. W. J. Gadella, P. B. Dhonukshe, C. J. F. Van Noorden, E. M. M. Manders, *Nat. Biotechnol.* **2007**, *25*, 249–253.
[10] P. T. Vernier, Z. A. Levine, Y. H. Wu, V. Joubert, M. J. Ziegler, L. M. Mir, D. P. Tieleman, *PloS one* **2009**, *4*, e7966.
[11] D. M. Engelman, T. A. Steitz et al., *Ann. Rev. Biophys. Biophys. Chem.* **1986**, *15*, 321-53.
[12] a) A. Wright, W. A. Bubb, C. L. Hawkins, M. J. Davies, *Photochem. Photobiol.* **2002**, *76*, 35–46; b) P. K. J. Kinnunen, K. Kaarniranta, A. K. Mahalka, *Biochim. Biophys. Acta Biomembr.* **2012**, *1818*, 2446–2455.
[13] I. Meerovich, N. Muthukrishnan, G. A. Johnson, A. Erazo-Oliveras, J. P. Pellois, *Biochim. Biophys. Acta Gen. Subj.* **2014**, *1840*, 507–515.
[14] M. A. de Ménorval, L. M. Mir, M. L. Fernandez, R. Reigada, *PloS one* **2012**, *7*, e41733.
[15] R. Notman, M. Noro, B. O'Malley, J. Anwar, *J. Am. Chem. Soc.* **2006**, *128*, 13982–13983.
[16] H. Melkonyan, C. Sorg, M. Klempt, *Nucleic Acids Res.* **1996**, *24*, 4356–4357.

Received: September 19, 2016
Published online: November 2, 2016

